Mo Bi Tec

Grow'n'Glow: The GFP One-Hybrid System

Product Information and Instructions
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Mo Bi Tec
MOLECULAR BIOLOGISCHE TECHNOLOGIE

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The Grow'n'Glow system was developed by Dr. Robert S. Cormack and Dr. Imre E. Somssich at the Max-Planck-Institut für Züchtungsforschung, Cologne, Germany. The system is patented.

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1. Introduction

1.1. General

The Grow'n'Glow GFP One-Hybrid Kit isolates genes for proteins that bind a specific DNA element of interest (Strubin *et al.*, 1995 and Luo *et al.*, 1996*).* In addition to finding novel DNA binding proteins, the one-hybrid system can be used to investigate the bases and amino-acids involved in specific DNA-protein interactions. Proteins can be found that bind to any short DNA element of interest. The Grow'n'Glow system offers maximal sensitivity because detection of the DNA-protein interaction occurs *in vivo*, where proteins are more likely to be in their native conformation.
Use of the Green Fluorescent Protein (GFP) reporter eliminates the time-consuming β-galactosidase lift assays. The particular GFP variant expressed by the GFP reporter plasmid, GFPuv, has the same excitation and emission maxima as wild-type GFP, but is 18 times brighter than the wild-type variant (Crameri *et al.*, 1996). It is easily detected by placing the plate (without the lid!) with the yeast colonies on a standard 300 nm UV transilluminator or, better, under a UV hand lamp in a darkroom. With this sensitive variant, positive colonies with DNA binding proteins glow a bright green, which is easily seen by eye under UV illumination.

Expression of fusion proteins by the prey plasmid is controlled by the GAL1 upstream activation sequence. In yeast with an intact galactose regulatory system, the GAL1 activation sequence is induced by galactose and repressed by glucose. This regulation provides a mean to prevent expression of library/ activation domain fusions until the actual screening, which avoids any potentially toxic library proteins affecting growth of the yeast. These sort of toxic proteins would be missed during screening in a non-inducible system.

For the Grow'n'Glow One-Hybrid assay, prepare a yeast reporter plasmid containing the sequence of a specific bait element upstream of the reporter gene GFP. For this purpose, at least three tandem copies of a known bait element are required. The bait elements are then inserted upstream of the reporter gene promoter of the GFP reporter vector using the available unique restriction enzymes (chapter 4.1.1.). Then, this construct (reporter-bait vector) is transformed into yeast cells and the transformants are selected by their marker gene. To screen a library for a gene encoding a DNA binding protein of interest, the user transforms the reporter-bait strain with a Grow'n'Glow library of fusions between the bait-independent activation domain and potentially bait-specific DNA binding proteins. The transformed yeast cells are then plated on selective medium plates. If a Grow'n'Glow library hybrid protein interacts with your bait element, the transcription of the GFPuv gene is activated. The interaction can be

verified under UV-light in a darkroom where colonies with DNA protein interactions will fluoresce green. Our experience is that the Grow'n'Glow GFP One-Hybrid System has a low incidence of false positives.
Next, Grow'n'Glow library plasmids are isolated from the positive transformants and characterized by sequencing. Finally, DNA binding should be confirmed by independent methods.
The Grow'n'Glow GFP One-Hybrid System can also be used to map the DNA binding domain of a known DNA binding protein. The difference to the procedure described above is, that instead of transforming the reporter-bait strain with a library, activation domain fusions are prepared with gene segments corresponding to specific domains of the known DNA binding protein. These constructs are transformed into the reporter-bait strain. The GFPuv reporter is used and the GFPuv activity can be quantified using a liquid assay (commercially available).

1.2. Background Information

The yeast one-hybrid system (Wang and Reed, 1993), a modified version of the two-hybrid system, rapidly identifies DNA binding proteins from either cDNA libraries or known gene sequences. The one-hybrid system exploits the bi-modular structure of eukaryotic transcriptional activators. A DNA binding element of interest is cloned into the promoter region controlling expression of the GFPuv reporter in the GFP reporter vector. A gene encoding a potential DNA binding protein is cloned in the prey plasmid so that it is expressed as a fusion to a B42 activator peptide. Interaction of the DNA binding protein with the DNA element brings the B42 activator peptide into a position that initiates transcription of the GFPuv reporter gene. Thus, colonies with DNA binding proteins that interact with the DNA element of interest are glowing brightly green, which is easily seen by eye under UV illumination (300 nm).

1.3. Green Fluorescent Protein (GFP)

The GFP of the jellyfish *A. victoria* is activated *in vivo* by an energy transfer via the Ca^{2+}-stimulation of the photoprotein aequorin (Crameri *et al.*, 1996). The blue light generated by aequorin excites GFP and results in the emission of green light. GFP itself consists of 238 amino acids (Mr = 27 kDa) and is synthesized as an apoprotein in which post-translational formation of the chromophore occurs in an O_2-dependent manner independent of any other gene products (Chalfie *et al.*, 1994 and Cubitt *et al.*, 1995). It maximally absorbs light at 395 nm and has an emission peak at 509 nm. The nonsubstrate requirement for GFP activity makes this protein an attractive reporter for gene expression studies and

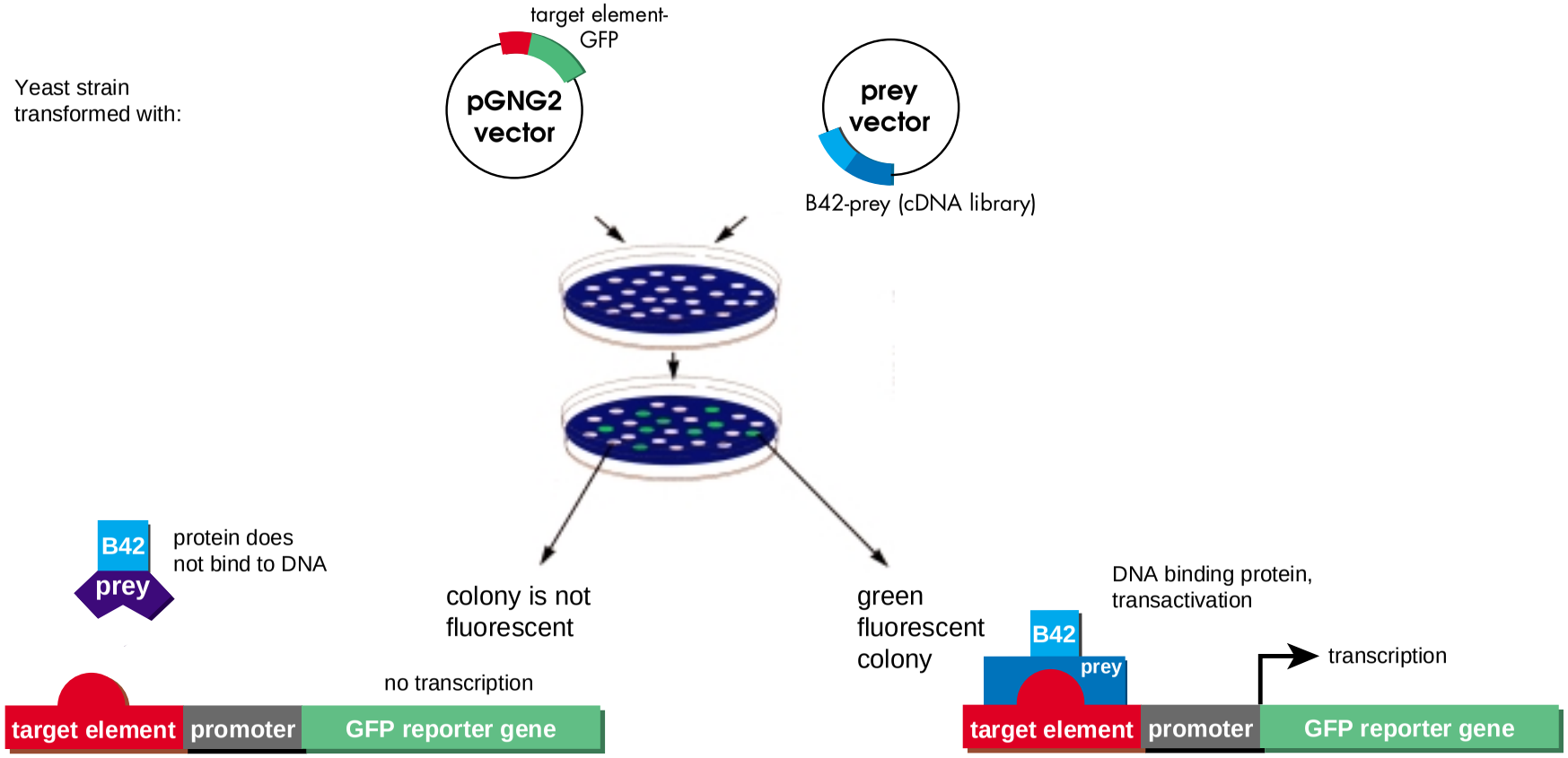
this utility was initially demonstrated in both prokaryotes (*Escherichia coli*) and eukaryotes (*Caenorhabditis elegans*), (Chalfie *et al.*, 1994). It has subsequently been used to monitor gene expression in many organisms including mouse (Chiocchetti *et al.*, 1997), Drosophila (Yeh *et al.*, 1995), zebrafish embryos (Meng *et al.*, 1997), Arabidopsis (Wilson *et al.*, 1991) and yeast (Niedenthal *et al.*, 1996). In addition to the non-invasiveness of GFP detection (long-wave UV light) the protein is very stable, non-toxic and resistant to photobleaching. These properties make GFP a viable alternative to traditional reporter genes such as β-galactosidase (LacZ), β-glucuronidase (GUS), chloramphenicol acetyltransferase (CAT) or firefly luciferase which require substrate for their detection. Several modifications of the wild-type GFP cDNA have been engineered with optimized codon usage, improved fluorescence activity and red- or blue-shifted variants with altered excitation maxima intended for fluorescence microscopy (Cubitt *et al.*, 1995). The *A. victoria* GFP variant, GFPuv, used in this one-hybrid system, is optimized for maximal fluorescence by UV-light excitation (360 - 400 nm) making it fluoresce 18 times brighter than wild-type GFP while retaining identical excitation and emission wavelength maxima.

2. Advantages of the Grow'n'Glow One-Hybrid System

- isolates genes for novel DNA binding proteins
- maps residues and regions responsible for DNA-binding
- non-invasive, chemical-free and cost-free assay
- positive colonies fluoresce bright green
- therefore, one-step selection of potential positives under UV light
- no time consuming β-galactosidase lift assays as essential in most conventional assays
- antibodies to HA-tag (fused downstream of B42) permit simple immunoprecipitation of potential positives
- finds potentially toxic protein by screening with galactose inducible expression libraries

3. Schematic Overview of the Grow'n'Glow One-Hybrid System

The One-Hybrid Approach:



4. Kit Components

Vectors	Primer	Host Strain
pGNG2*	GNGprimer	Yeast strain EGY48
pJG4-5*	5'-PREYprimer	
pGNG2-p53*	3'-PREYprimer	
pJG-4-5-p53*		

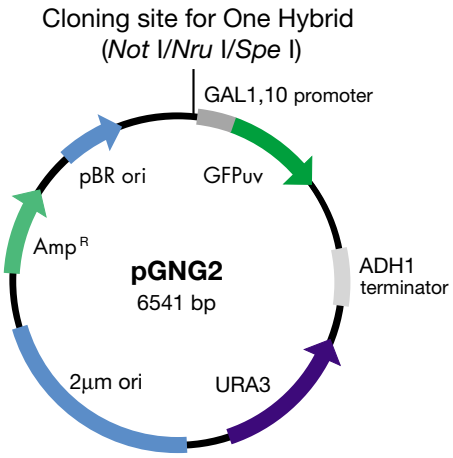
*Re-dissolve lyophilized vector DNA in TE-buffer (pH 8.0)

Order information see Appendix III.

4.1. DNA Vectors

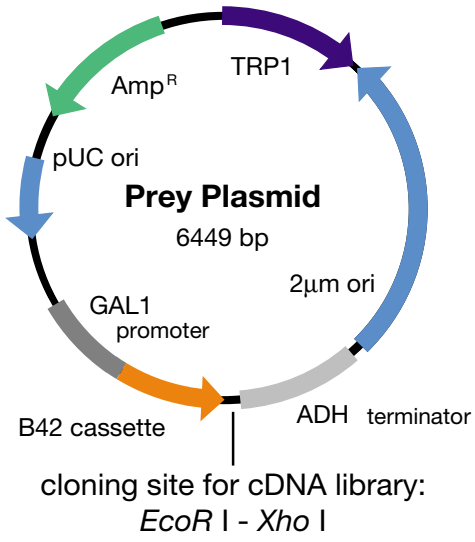
4.1.1. GFP Reporter Vector

The reporter plasmid pGNG2 is suited for one-hybrid screens with the reporter gene GFP. This GFPuv gene is driven by a GAL1,10 minimal promoter. The 6.51 kb plasmid contains a URA3 selectable marker, 2 µm origin to allow propagation in yeast, and the ampicillin resistance gene (Amp^R) and origin of replication (pBR ori) for propagation in *Escherichia coli*. pGNG2 has unique restriction sites for *Not* I, *Nru* I and *Spe* I suitable for cloning a DNA target element of interest for a one-hybrid screen.



4.1.2. Prey plasmid pJG4-5

The prey plasmid pJG4-5 (Crameri *et al.*, 1996) is used to express cDNAs or other coding sequences inserted into the *Eco*R I and *Xho* I sites as translational fusions to a cassette consisting of the SV40 nuclear localization sequence, the 88-residue acidic activator B42 (acid blob) and the HA (hemagglutinin) epitope tag. Fusion protein expression is controlled by the GAL1 inducible promoter, thus, transcription levels are very low in the presence of glucose and high with galactose. For selection in yeast, the vector contains the TRP1 (tryptophan) marker and the 2 µm origin of replication; for propagation in *E. coli* an ampicillin resistance (Amp^R) and pUC origin are present.



pJG4-5 polylinker:

5'-CCC GAA TTC GGC CGA CTC GAG AAG-3'

*Eco*R I *Xho* I

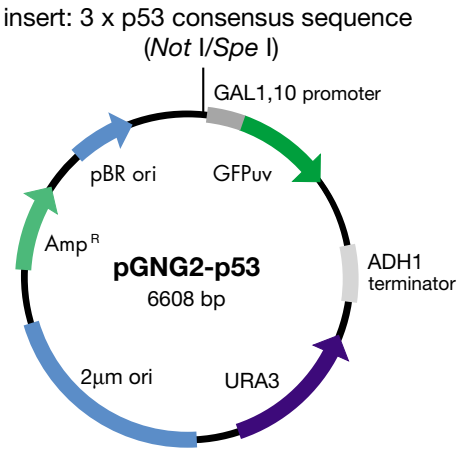
Polylinker of the vector indicating the open reading frame. The shown restriction endonucleases are only located in the polylinker.

4.1.3. Control Plasmids

GFP positive control vector pGNG2-p53:

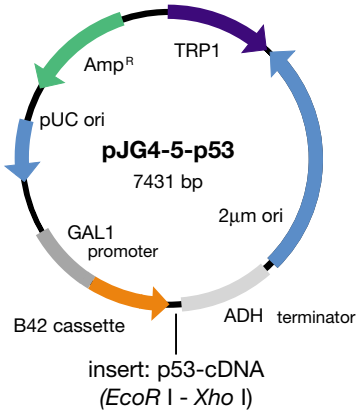
The GFP positive control reporter vector pGNG2-p53 consists of pGNG2 and 3 tandem repeats of the mouse p53 consensus sequence cloned into the *Not* I/*Spe* I sites of the pGNG2 MCS upstream of the Gal1,10 promoter.

pGNG2-p53 serves as positive control in combination with the prey control vector pJG4-5-p53 which carries the mouse p53-cDNA.



Prey control plasmid pJG4-5-p53:

The prey control plasmid pJG4-5-p53 consists of pJG4-5 and the sequence of the mouse p53-cDNA (cloned into the *Eco*R I/*Xho* I sites of pJG4-5) which is fused to the B42 activation domain.



4.2. Primer

4.2.1. GNGprimer

5′-GCC CAA TAC GCA AAC CGC CT- 3′

This sequence is situated 38 bp upstream of the unique *Not* I restriction site. Used to verify bait DNA introduced within the cloning sites of pGNG2 by sequencing the construct.

4.2.2. 5'-PREYprimer

5'-CTG AGT GGA GAT GCC TCC-3'

5'-forward primer used to determine the reading frame and identity of positive clones in pJG4-5; can be used with 3'-primer to amplify clone by PCR*.

4.2.3. 3'-PREYprimer

5'-GCC GAC AAC CTT GAT TG-3'

3'-primer used to determine the identity of positive clones in pJG4-5; can also be used with 5'-primer to amplify clone by PCR.

*PCR is a process covered by patents owned by Hoffmann-La Roche. Use of this process requires a licence

4.3. Yeast Strain

The provided yeast strain is a high-sensitivity strain and has the following genotype:

S. cerevisiae EGY48: MATα, trp1, his3, ura3, leu2::6 LexAop-LEU2.

It is frozen in YPD medium containing 15 % glycerol and can be stored indefinitely at -70°C.

5. Materials Required, but not Supplied

Note: The specific materials listed below are the ones we have tested in the Grow'n'Glow System. For order information see Appendix III. Similar items from other sources may be interchangeable.

1. Grow'n'Glow Yeast Growth media (see chapter 5.1.)

2. Grow'n'Glow Bacterial Growth media (see chapter 5.1.)

3. Ampicillin, Boehringer Mannheim order # 835 269

4. Grow'n'Glow Yeast Transformation kits (**MoBiTec** order # 2100-1 and # 2200-1)

5. Grow'n'Glow Yeast Plasmid Isolation kit (**MoBiTec** order # 2069-1)

5.1. Recipes for Media

For optimal results, we highly recommend to use the Grow'n'Glow Yeast and Bacterial Growth Media offered by **MoBiTec** (see chapter 5.1.1. and 5.1.2.), which are optimized for one-hybrid systems. Rich medium YPD and the standard Wickerham yeast nitrogen base with carbon source optimized for *S. cerevisiae* can be added to water and autoclaved without the need to make concentrated solutions of vitamins, trace elements, salts or carbon sources.
The powder dropout base formulation which is called Drop Out Base (DOB) medium or DOBA (with Agar) is a standard Wickerham yeast nitrogen base with a carbon source. A complete supplemented synthetic defined medium is easily made by mixing two powders, DOB (or DOBA) and Complete Supplement Mixture (CSM). The formulation of CSM is a dropout supplement for virtually all strains of *S. cerevisiae* containing different combinations of amino acids, adenine and uracil. Cells grow vigorously in DOB supplemented with CSM. These media are very easy to handle and are delivered as powder. Some of our media are available in small bags with the appropriate amount of powder for 0.5 litre medium (10 bags per package are sufficient for 10 x 0.5 litre medium). Just add water and autoclave - ready!

For order informations see Appendix III.

5.1.1. Grow'n'Glow Yeast Growth Media

Notes: *DOB = Drop Out Base*

DOBA = Drop Out Base with Agar

"-TRP" signifies medium lacks tryptophan

"-URA" signifies medium lacks uracil

a) YPD and YPD agar (rich medium; 20 g peptone, 10 g yeast extract, 20 g glucose, pH 6.5, 17 g agar per litre):

YPD Broth:

Pour the entire content of the YPD broth bag (**MoBiTec** order # 4001-1) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Ready for use.

YPD Agar:

Pour the entire content of the YPD agar bag (**MoBiTec** order # 4001-2) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Pour into plates. Ready for use.

b) DOB media and DOBA agar with glucose or galactose/raffinose (selective medium; 1.7 g yeast nitrogen base, 5 g ammonium sulfate, 20 g glucose or 20 g galactose and 10 g raffinose, 17 g agar per litre):

DOB -URA (glucose):

Pour the entire content of a DOB glucose bag (**MoBiTec** order # 4025-1) and 0.385 g CSM -URA (**MoBiTec** order # 4511-2) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Ready for use.

DOBA -URA (glucose):
Pour the entire content of a DOBA glucose bag (**MoBiTec** order # 4026-1) and 0.385 g CSM -URA (**MoBiTec** order # 4511-2) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Pour into plates. Ready for use.

DOB -TRP -URA (glucose):

Pour the entire content of a DOB glucose bag (**MoBiTec** order #4025-1) and 0.36 g CSM -TRP -URA (**MoBiTec** order # 4520-5) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Ready for use.

DOBA -TRP -URA (glucose):

Pour the entire content of a DOBA glucose bag (**MoBiTec** order # 4026-1) and 0.36 g CSM -TRP -URA (**MoBiTec** order # 4520-5) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Pour into plates. Ready for use.

DOB -TRP -URA (galactose/raffinose):

Pour 18.4 g DOB Gal/Raf (**MoBiTec** order # 4025-2) and 0.36 g CSM -TRP -URA (**MoBiTec** order # 4520-5) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Ready for use.

DOBA -TRP -URA (galactose/raffinose):

Pour 18.4 g DOBA Gal/Raf (**MoBiTec** order # 4026-2) and 0.36 g CSM -TRP -URA (**MoBiTec** order # 4520-5) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Pour into plates. Ready for use.

5.1.2. Grow'n'Glow Bacterial Growth Media

a) LB Medium:

Pour the entire content of a LB medium bag (**MoBiTec** order # 3002-1) into a 0.5 l flask add 500 ml H_2O. Autoclave. Cool to at least 37°C. Ready for use.

b) LB Agar Medium:

Pour the entire content of a LB agar medium bag (**MoBiTec** order # 3002-2) into a 0.5 l flask add 500 ml H_2O. Autoclave. Cool to at least 55°C. Pour into plates.

c) LB Amp Medium (ampicillin selection):

Cool the LB Medium above to at least 55°C and add 2 ml of 50 mg/ml ampicillin (in distilled water, filter-sterilized) per litre of medium. Mix.

d) LB Amp Agar Medium (ampicillin selection):

Cool the LB Agar Medium above to 55°C and add 2 ml of 50 mg/ml ampicillin (in distilled water, filter-sterilized) per litre of medium. Mix. Pour into plates.

6. Growth and Maintenance of Yeast

The yeast strain (*Saccharomyces cerevisiae*; see chapter 4.3.) in our kit is provided in YPD medium with 15 % glycerol and can be maintained indefinitely at -70°C.

To recover the yeast strain from the frozen glycerol stock, scrape a small amount of frozen cells from the frozen stock with a sterile loop or wooden stick and streak them onto a YPD plate. Incubate plate at 30°C for 1 - 3 days until colonies appear. Seal this working stock plate with Parafilm and store at 4°C. Propagate additional cultures only from isolated single colonies on this plate. You should restreak this plate every 2 - 3 weeks.

Note: The cells may have settled to the bottom of the tube before the stock was frozen. If this happens, thaw the frozen culture on ice and vortex it before restreaking. The stock can be refrozen.

Healthy yeast colonies grow to > 2 mm in diameter. Avoid small white colonies (< 1 mm) which are cells with spontaneous mutations (1 - 2 %) that eliminate mitochondrial function (Holm, 1993) when you inoculate a culture.

General remarks: Yeast can be grown on plates or in liquid culture, like *E. coli*. **However, antibiotics, which work on *E. coli*, do not work on yeast, making good sterile technique mandatory when working with yeast.** The optimum growth temperature for yeast is 28 - 32°C. The growth rate is relatively rapid, with a doubling time of 90 - 120 minutes. Budding yeast is very amenable to genetic and molecular biological methods due to its ability to be transformed by foreign DNA and its highly efficient system of homologous recombination.

General knowledge: Users should be familiar with basic molecular biology and microbiological techniques (Ausubel *et al.*, 1997; Sambrook *et al.*, 1989).

7. Grow'n'Glow One-Hybrid System Protocol

7.1. Reporter-Bait Constructs

7.1.1. GFP Reporter Vector and Prey Plasmid

In order to screen a cDNA for DNA binding proteins using the Grow'n'Glow One-Hybrid System a true or putative bait element has to be identified. The bait element must be precisely defined using, for example, deletion and/or point mutation analysis. Prepare a construct composed of three or more tandem copies of your bait regulatory element bordered by restriction sites and insert it upstream of the reporter gene in the multiple cloning site of the GFP reporter

plasmid. This may alter the level of background GFPuv expression. Therefore, constructs should always be tested for GFPuv expression under UV-light. The prey plasmid carries the GAL1 promoter which is induced by galactose and inhibited by glucose. This feature ensures that even toxic proteins can be expressed.

7.1.2. Bait Element: Tandem Copy Synthesis

At least three tandem copies of the bait element should be inserted upstream of the reporter gene (Ghosh *et al.*, 1993).

Various methods (e.g. Liaw, 1994) are available to generate tandem copies, however, we have found the most convenient and reliable method for generating them to be oligonucleotide synthesis, since well-defined regulatory elements are usually smaller than 20 bp.

1. Two antiparallel oligonucleotides are designed, one representing the sense strand and the other its antisense complement. The sense strand should consist of at least three tandem copies of the bait element with a different restriction site on each end. When the two strands are annealed, the resulting double-stranded DNA will have a different overhang at each end for directional cloning into the reporter plasmid's multiple cloning site.
2. Synthesize both strands without 5'-phosphates.

7.1.3. Tandem Copies of Bait inserted Upstream of GFP Reporter Gene

7.1.3.1. Reagents and Materials Required

- Bait element: Sense- and antisense-strand oligonucleotide
- Competent *E. coli* cells (Sambrook *et al.*, 1989)
- T4 DNA ligase
- 10x T4 ligation buffer (Sambrook *et al.*, 1989; or the buffer provided with the commercial enzyme)
- *Not* I, *Nru* I, *Spe* I, *Xba* I and other restriction enzymes
- LB/amp plates
- 50 mM NaCl (autoclaved)
- Materials for purifying plasmid from *E. coli* transformants (Grow'n'Glow Yeast plasmid isolation kit can be used as well; **MoBiTec** order # 2069-1)

7.1.3.2. Annealing of Sense/Antisense Oligonucleotides

Construction of the bait element for cloning into the GFP reporter vector:

1. For each construct planned, mix 0.1 µg of sense-strand and 0.1 µg of antisense-strand oligonucleotide of the bait element in 10 µl of 50 mM NaCl.
2. Anneal the bait element oligonucleotides by heating at 70°C for 5 minutes and then slowly cooling to room temperature (~ 30 minutes).
3. Completely digest 0.1 µg of the GFP reporter plasmid in a 20 µl double digest using an appropriate pair of restriction enzymes such as those recommended below. Incubate at 37°C for 2 hours, or as directed by the enzyme manufacturer.

   • GFP reporter: *Not* I, *Nru* I and *Spe* I, (*Xba* I).

   Electrophorese 2 µl of the digest on an 1 % agarose gel to confirm that the plasmid has been efficiently linearized.
4. Mix 5 µl of the GFP reporter digested plasmid, with 1 µl of annealed oligo. Add 4 µl of H_2O.
5. Add 1.2 µl of 10x T4 ligation buffer and 0.8 µl (at least 0.8 units) of T4 DNA ligase, and incubate at room temperature for 4 hours.

   Note: Since the molar ratio of oligonucleotide to vector is 100 :1 or greater, no gel purification to remove the stuffer fragment is required.
6. Transform competent *E. coli* cells with each construct using a standard method (Sambrook *et al.*, 1989).
7. Plate transformants on LB/amp plates, and incubate at 37°C overnight.
8. Prepare plasmid using any standard method that yields highly pure DNA (Sambrook *et al.*, 1989) or use the **MoBiTec** plasmid isolation kit (order # 2069-1) for yeast which can also be used for plasmid DNA in *E. coli*. Check for inserts by sequencing across the junctions with the GFP reporter primer.

7.2. Construction of the GFP Reporter-Bait Strain

We recommend a two-step transformation procedure instead of co-introducing the GFP reporter-bait and prey plasmid-library vectors. A small-scale transformation procedure is performed to transform the GFP reporter-bait construct into the EGY48 strain.

Use the Grow'n'Glow High Efficiency Yeast Transformation kit (**MoBiTec** order # 2200-1) or the Grow'n'Glow Fast and Easy Yeast Transformation kit (**MoBiTec** order # 2100-1), which are optimized for two- and one-hybrid systems. The

Grow'n'Glow Yeast Transformation kits contain all the solutions required for transformation.

Use the protocol supplied with the Grow'n'Glow Yeast Transformation kits using 200 ng of plasmid DNA for transformation or the procedure described in Appendix I.

7.3. Screening a Grow'n'Glow cDNA Library for DNA Binding Protein Genes

7.3.1. Reagents and Materials Required

- DOB -URA and DOB -TRP -URA (Glu) medium (chapter 5.1.1.)
- DOB -TRP -URA (Gal/Raf) agar plates medium (chapter 5.1.1.)
- 15 sterile 245 x 245 x 25 mm Nunc Bio-Assay Dishes (Nunc, # 240835A)
- Appropriate sterile tubes and flasks

  Notes: a) Prepare the selection media, and pour the required number of agar plates in advance. You will need 15 Petri dishes 22 x 22 cm DOB -TRP -URA at 37°C for the transformation.

  b) Allow DOB agar plates to dry (unsleeved) at room temperature for 2 - 3 days or at 30°C for 3 hours prior to plating transformation mixtures.

- EGY48 yeast strain
- Grow'n'Glow library plasmid DNA in solution. Your prey library should have at least 10^6 clones.

  For information on constructing your own libraries, see Vojtek, 1993; Durfee *et al.*, 1993; and Dalton and Treisman, 1992.

- Herring testes carrier DNA (Sigma)
- Sterile 1x PEG/LiAc solution (prepare immediately prior to use from 10x stocks)
- 100 % DMSO (dimethylsulfoxide; Sigma # D-8779)
- 1x TE-buffer; prepare from 10x TE-buffer (100 mM Tris pH 7.5, 10 mM EDTA)
- Sterile glass rod, bent pasteur pipette, or 5 mm glass beads for spreading transformation mixtures on plates.

  Note: The Yeast Transformation Systems (**MoBiTec** order # 2200-1 or # 2100-1) contains all the solutions required for yeast transformation optimized for use in the Grow'n'Glow Two- and One-Hybrid Systems.

7.3.2. Large-Scale Yeast Transformation

This step is very critical. The number of recombinants transformed with the prey clones should be as high as possible. We therefore highly recommend the use of the Grow'n'Glow High Efficiency Yeast Transformation kit (**MoBiTec** order # 2200-1) for obtaining the best results (contains an optimized library transformation procedure). Otherwise, you can try the up-scaled procedure below.

This protocol is scaled for screening > 1x 10^6 independent clones**.**

1. Inoculate several colonies (4 - 5) of the transformed GFP reporter-bait strain into 1 ml of DOB -URA (Glu) medium.
2. Vortex vigorously for 2 minutes to disperse any clumps.
3. Transfer this cell suspension into a flask containing 50 ml of DOB -URA (Glu) medium.
4. Incubate at 30°C for 16 - 18 hours with shaking at 250 rpm to stationary phase (OD_{600} > 1.5).
5. Transfer enough overnight culture to produce an OD_{600} = 0.2 - 0.3 into 300 ml of DOB -URA (Glu) medium.
6. Incubate at 30°C for 3 h with shaking at 230 rpm. The OD_{600} will be 0.4 - 0.5.
7. Centrifuge the culture in 50 ml tubes at 2000x g for 5 minutes at room temperature.
8. Discard the supernatant and vortex to resuspend each cell pellet in 25 ml of TE-buffer.
9. Pool the cells into one tube.
10. Centrifuge the cells again at 2000x g for 5 minutes at room temperature.
11. Discard the supernatant and resuspend the cell pellet in 1.5 ml of freshly prepared sterile 1x TE/LiAc. Mix well by vortexing.
12. In a sterile 50 ml tube, add 20 - 40 µg of prey plasmid library and 5 mg of carrier DNA, and mix well.
13. Add 1 ml of competent cells to the step 12 DNA mixture, and mix well by vortexing.

    Note: If cells are not mixed well, transformation efficiency may decline.
14. Add 6 ml of sterile 1x PEG/LiAc to the transformation mixture.
15. Mix well by inverting at least ten times.
16. Incubate at 30°C for 30 minutes with shaking at 200 rpm.

17. Add 700 µl of DMSO and mix well by gentle inversion. Do not vortex.
18. Heat shock for 15 minutes in a 42°C water bath. Occasionally invert carefully to mix.
19. Chill on ice for 2 minutes.
20. Centrifuge at 2000x g for 5 minutes at room temperature, and remove supernatant.
21. Resuspend cells in ~25 ml of TE-buffer.

7.3.3. Plating the Transformation Mixture

1. Plate 1ml of the transformation mixture on each 245 x 245 mm plate (15 plates total) containing DOB -TRP -URA (Gal/Raf) agar (chapter 5.1.1.) which stimulate the expression of the fusion proteins of the prey plasmid. Spread the cells immediately after pipetting them onto the plate.
2. Incubate at 30°C for 2 - 4 days.
3. Place the plates (without the lid) under an UV-light (365 nm) in a darkroom. Localize and isolate all colonies emitting green light using a wooden stick and streak onto a new fresh 100 mm DOB -TRP -URA (Gal/Raf) plate.
4. Incubate the isolated and restreaked putative positives at 30°C for 2 - 4 days.
5. Place the plates (without the lid) under an UV-hand-lamp in a darkroom. Identify the colonies emitting green light and inoculate these in 5 ml DOB -TRP -URA (Glu) medium. Incubate at 30°C for 3 hours with shaking at 230 rpm. Continue with yeast plasmid purification (see chapter 7.3.4.).

7.3.4. Isolating the Prey Plasmid from Putative Positive Yeast Colonies

We recommend the Grow'n'Glow Yeast Plasmid Isolation kit (**MoBiTec** order # 2069-1) providing the reagents for a fast and simple protocol for isolating plasmids from yeast. This procedure will provide plasmid DNA suitable for PCR and *E. coli* transformations. Otherwise you can use the methods described by Ausubel *et al.*, 1997.

We recommend that the identity of the selected clones is confirmed by independent methods. First, *E. coli* cells are transformed with plasmids isolated from yeast as described above.

1. Isolate plasmids from four *E. coli* colonies using any method that produces

highly pure DNA. Use the Grow'n'Glow Yeast Plasmid Isolation kit (**MoBiTec** order # 2069-1) for this purpose or consult Sambrook *et al.*, 1989.

2. Identify the *E. coli* colonies containing the prey plasmid, by restriction enzyme analysis, by sequencing with the 5'-PREYprimer or by colony PCR amplification using the 5'-PREYprimer and 3'-PREYprimer (see next chapter).

   Note: PCR using the 5'-PREYprimer and 3'-PREYprimer with the prey vector (carrying no insert) as template DNA results in a 125 bp DNA fragment.

7.3.5. Prey Plasmid Identification by PCR*

1. Inoculate 4 colonies from each plate in 2 ml LB-Amp medium. Grow overnight at 37°C.
2. 5 µl culture are transferred to a PCR tube, centrifuged 5 seconds at maximal speed. Discard supernatant. Add 30 µl PCR-mix and mix.

PCR mix:	20 pmole	5'-PREYprimer
	20 pmole	3'-PREYprimer
	3 µl	10x dNTP (2.5 mM dNTP)
	3 µl	10x Taq DNA polymerase buffer
	1 µl	Taq DNA polymerase (2.5 U/µl)
	H_2O up to 30 µl.	

3. Run the reaction in a thermal-cycler as follows:

1 cycle	2 minutes 95°C
20 cycles	30 seconds 95°C 30 seconds 60°C 120 seconds 72°C
1 cycle	5 minutes 72°C

4. Load the PCR reactions on an agarose gel. Clones with an amplified insert contain a prey plasmid with an insert.
5. Go back to the cultures (see step 1.). Purify the target plasmid from positive clones with an insert using an available plasmid purification method.
6. Sequence the target plasmid insert with the 5'-PREYprimer.
7. Use the DNA sequence to search against a database of choice.

* PCR is a process covered by patents owned by Hoffmann-La Roche. Use of this process requires a licence.

7.4. Confirmation of DNA Binding Activity

Although none of the tests suggested below is independently conclusive, the results should provide enough convincing evidence together to support whether the Grow'n'Glow library plasmid encodes a DNA binding protein.

1. You may want to perform a positive control experiment, if a non-binding mutant of your target element is available. First prepare a mutant type construct in the GFP reporter plasmid otherwise identical to your original target reporter construct.

   Then transform the construct into EGY48, and transform the new reporter strain with the candidate prey library plasmid. Fluorescing colonies should result from transcriptional activation using the wild type but not mutant target, indicating that you have identified a sequence specific DNA binding protein (see Li and Herskowitz, 1993, for an example).

2. Sequence the positive library clones with the 5'-PREYprimer and compare the sequence with that of other DNA binding proteins in GenBank, EMBL or other databases.

   a. In case your sequencing results reveal a peptide below ten amino acids fused to the B42 activation domain (or no fusion peptide at all) keep sequencing beyond the stop codon. Another (larger) open reading frame (ORF) for a peptide that interacts with the target elements in your reporter strain may be found, which functions as a transcriptional activator.
   In yeast genomic libraries, where intercistronic regions are very short, nontranslated gaps upstream of ORF inserts are most commonly found. Due to the cloning of a portion of the 5'-untranslated region of the mRNA along with the coding region in the cDNA, such gaps can also occur in cDNA libraries. If the library was built in a high-level expression vector (such as the prey plasmid), a western blot analysis will reveal the presence (and size) of a B42 activaton domain fusion protein.

   b. It has been observed that the positive library clone is transcribed in the reverse orientation from a cryptic promoter within the ADH1 terminator (Chien *et al.*, 1991). Your sequencing results would then fail to reveal any ORF in frame with the B42 activator coding region. The expressed proteins apparently function as transcriptional activators as well as interact with the target elements.

   c. In some cases, two different ORFs may be expressed as a fusion with the B42 activation domain even though a nontranslated gap comes between them due, for example, to occasional translational read-through.

3. If you have a library clone that you believe encodes a transcriptional activator, check if the protein has a nuclear localization tag as many DNA transcrip-

tional activators have. You may achieve a crude result by observing the predicted amino acid sequence and comparing it to known nuclear localization sequences, but of course the best evidence for a nuclear localization domain is functional evidence. For example, this could be analyzed by transferring the insert to an expression vector that will generate a fusion of the protein with a cellular localization tag, such as the green fluorescent protein.

4. Gel-shift DNA binding assay with protein extracts. When preparing yeast protein extracts for use in an electrophoretic mobility shift assay (EMSA), use a procedure that will yield native proteins. The following procedure is summarized from Arndt *et al.*, 1987:

   a. Prepare an overnight culture of the yeast transformant in DOB -TRP -URA (Glu) medium (to keep selection on the prey library plasmid). The OD_{600} should be ~1.0.

   b. Centrifuge 100 ml of the culture. Discard the supernatant and resuspend the pellet in 400 µl of extraction buffer: 0.1 M Tris-HCl (pH 7.5), 0.2 M NaCl, 0.01 M β-mercaptoethanol, 20 % glycerol, 5 mM EDTA and 1 mM PMSF.

   c. Transfer cell suspension to a pre-chilled glass tube and add an equal volume of glass beads. Place sample on ice and vortex vigorously for ~ 10 minutes (not including pause times to allow for sample cooling).

   d. Allow glass beads to settle, then transfer all available liquid to another pre-chilled glass tube.

   e. Add 200 µl of extraction buffer to the liquid, add an equal volume of glass beads and vortex again as described above.

   f. Separate the liquid from the glass beads by centrifugation.

      Note: One way to do this is to punch a pinhole in the microcentrifuge tube and nest this tube inside another tube before adding the sample. Upon centrifuging, the liquid will flow through to the collection tube, leaving the beads behind.

   g. Freeze the liquid quickly in liquid nitrogen and store it at -70°C.

   h. The protein yield is typically 10 - 20 mg/ml. Use 2 - 5 µl in the EMSA.

5. Perform *in vitro* translation and a DNA binding assay (Wu *et al.*, 1994). A comparison of the gel-shift results between the wild-type bait DNA element and a mutant bait DNA element may prove excellently instructive.

7.5. Troubleshooting Guide

7.5.1. Excessive Background Growth and Fluorescence on Library Screening Medium

Solution 1:

Check to make sure that you have prepared the selection medium correctly.

Solution 2:

You may have used too high amounts of the Grow'n'Glow prey plasmid for transformation. Perform a new transformation with lower amounts of the prey plasmid.

Solution 3:

Your inserted bait element may be interacting with yeast endogenous transcriptional activators. It may be necessary to redesign the target element and construct a new GFP reporter-bait plasmid.

7.5.2. Low Transformation Efficiency When Screening a Grow'n'Glow Library

The transformation efficiency should be at least 10^4 cfu/µg for the library transformation. If your library transformation efficiency is lower than this, try one or more of the following suggestions.

Solution 1:

Repeat the experiment using more of the prey library plasmid. Check the purity of the DNA and, if necessary, re-purify it by ethanol precipitation before using it again. If you are not already doing so, we strongly recommend using the pre-tested and optimized carrier DNA, which is available as part of the Grow'n'Glow Yeast Transformation kits (**MoBiTec** order # 2100-1or 2200-1).

Solution 2:

Repeat the transformation, this time including a "recovery" period after the heat shock. To provide a recovery period, perform the transformation as described, but add the following steps after step 20:

1. Resuspend cells in 50 ml of DOB -TRP -URA (Glu) medium. Divide cell suspension into two 50 ml tubes.
2. Incubate cells at 30°C for 1 h with shaking at 230 rpm.
3. Centrifuge at 2000x g for 5 minutes at room temperature. Remove supernatant.

7.5.3. False Negative Results

A protein that normally interacts *in vivo* does not interact with the bait element.

Solution 1:

If one of the following situations is occurring, it may interfere with the ability of the B42 activator hybrid proteins to interact with the bait element:

1. The hybrid protein cannot be localized to the yeast nucleus (see van Aelst *et al.*, 1993, for one example).
   In these cases, it may help to construct hybrids containing different domains of the DNA binding protein. For example, to study proteins that normally do not localize to the nucleus, it may be necessary to generate mutant forms of the protein that can be transported across the nuclear membrane.
2. The hybrid protein folds improperly.
3. The B42 activator occludes the site of interaction.
4. The hybrid proteins are not stably expressed in the host cell.

Solution 2:

If according to the control in chapter 7.3.4. the transformation efficiency is too low, you may not be screening a sufficient number of library co-transformants. This can be critical, especially if a rare transcript in the source tissue encodes the interacting protein of interest. See chapter 7.5.2. above for tips on improving transformation efficiency.

8. Literature

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Appendix I:

Small-Scale Yeast Transformation

Reagents and Materials Required

- YPD-Medium (chapter 5.1.1.a)
- Appropriate sterile tubes and flasks
- Appropriate DOB -URA agar plates (chapter 5.1.1.b)
- Yeast strain: EGY48
- Herring testes carrier DNA (Sigma)
- Sterile 1x PEG/LiAc solution (Prepare immediately prior to use from 10x stocks)
- 100 % DMSO (Dimethylsulfoxide; Sigma # D-8779)
- 1x TE-buffer; prepare from 10x TE-buffer (100 mM Tris pH 7.5, 10 mM EDTA)
- Sterile glass rod, bent pasteur pipette, or 5 mm glass beads for spreading transformation mixtures on plates.

Tips for a Successful Transformation

1. Fresh (1 - 3 days old) colonies will give best results for liquid culture inoculation. A single large (2 - 3 mm) colony is used for the inoculum. Scrape the entire colony into the medium. If colonies on the stock plate are smaller than 2 mm, scrape several colonies into the medium (see also chapter 7!).
2. If the overnight or 3 hours cultures are visibly clumped, disperse the clumps with vigorous vortexing before using them in the next step.
3. When you are collecting cells by centrifugation, a swinging bucket rotor results in better recovery of the cell pellet.
4. For the highest transformation efficiency (as is necessary for library screening), use competent cells within 1 hour of their preparation. If necessary, competent cells can be stored (after step 10) at room temperature for several hours with a minor reduction in competence.
5. To obtain an even growth of colonies after plating, continue to spread the

transformation mixtures over the agar surface until all liquid has been absorbed.

Small-Scale Yeast Transformation Protocol

This protocol is for small-scale transformation, obtaining 100 - 1000 transformants.

1. Inoculate several colonies of the yeast strain 2 - 3 mm in diameter, into 1 ml of YPD medium.
2. Vortex vigorously for 2 minutes to disperse any clumps.
3. Transfer 100 µl of this cell suspension into a flask containing 5 ml of YPD medium.
4. Incubate at 30°C for 16 - 18 hours with shaking at 250 rpm until stationary phase is reached (OD_{600} > 1.5).
5. Transfer enough overnight culture to produce an OD_{600} = 0.2 - 0.3 into 50 ml of YPD medium.
6. Incubate at 30°C for 3 hours with shaking at 230 rpm. The OD_{600} will be 0.5 ± 0.1.
7. Centrifuge the culture in 50 ml tubes at 2000x g for 5 minutes at room temperature.
8. Discard the supernatant and vortex to resuspend each cell pellet in 5 ml of TE-buffer.
9. Centrifuge the cells again at 2000x g for 5 minutes at room temperature.
10. Discard the supernatant and resuspend the cell pellet in 300 µl of freshly prepared, sterile 1x TE/LiAc. Mix well by vortexing.
11. In a sterile 50 ml tube, add 0.2 - 1 µg of GFP reporter-bait plasmid and 50 µg of carrier DNA and mix well. (Very important that the carrier DNA is denaturated several times by boiling in a water bath.)
12. Add 250 µl of competent cells to the step 11 DNA mixture, and mix well by vortexing.

    Note: If cells are not mixed well, transformation efficiency may decline.
13. Add 1.2 ml of sterile PEG/LiAc to the transformation mixture.
14. Vortex at high speed for 10 seconds to mix well.
15. Incubate at 30°C for 30 minutes with shaking at 200 rpm.
16. Add 100 µl of DMSO and mix well by gentle inversion. Do not vortex.

17. Heat shock for 15 minutes in a 42°C water bath. Swirl occasionally to mix.
18. Chill on ice for 2 minutes.
19. Centrifuge at 2000x g for 5 minutes at room temperature, and remove supernatant.
20. Resuspend cells in ~200 µl of TE-buffer.
21. Plate the transformation mixture onto an 85 mm DOB -URA agar plate.
22. Incubate at 30°C for 2 - 3 days. Store the plate at 4°C, when growth is achieved.

Appendix II

Competent *E. coli* Cells and Transformation

Competent Cells

1. Inoculate one colony of *your E. coli* strain in 5 ml SOB medium.

   Note: Use a freshly streaked plate with your *E. coli* strain of choice.
2. Incubate 250 ml SOB with 2.5 ml of the overnight culture. Incubate at 37°C while shaking at 200 rpm. Measure the OD_{550} after 2 - 2.5 hours. Once a density of 0.4 - 0.5 (OD_{550}) is reached place cells on ice for 15 minutes. It is very important to keep the temperature at 0°C unless otherwise stated.
3. Pellet cells by centrifugation. Spin 10 minutes at 6000 rpm (3000 g) at 4°C.
4. Discard the supernatant.
5. Resuspend in ice-cold 80 ml RF I-buffer. Place on ice for 20 minutes.
6. Spin 6000 rpm for 10 minutes at 4°C.
7. Discard the supernatant. Resuspend the pellet in ice-cold 20 ml RF II-buffer.
8. Aliquot the cells in 220 µl aliquots on ice. Quick-freeze immediately in liquid nitrogen or dry-ice/methanol. Store at -80°C until use.

Buffers

SOB:

500 ml: 2 % (w/v) Bacto-Tryptone or peptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$. Autoclave.

RF I buffer:

400 ml: 100 mM RbCl (rubidium chloride), 30 mM KAc, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % Glycerol. Adjust to pH 5.8 with 0.2 M acetic acid. Autoclave.

RF II-buffer:

100 ml: 10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, distilled H_2O to approximately 75 ml. Adjust to pH 6.8 with 1 M NaOH. Add H_2O up to 100 ml. Autoclave.

Transformation of Recombinant Plasmids into *E. coli*

Note on controls: In addition to the experimental transformations described below, we recommend that you perform a negative control transformation with no DNA and a positive control transformation with 0.1 ng of an intact plasmid such as pBR322 or pUC19 (0.1 ng pUC19 should result in 100 - 1000 colonies).

1. Thaw the competent *E. coli* cells on ice to obtain maximum efficiency.

   Caution: Once thawed, cells cannot be refrozen.
2. Place 5 µl plasmid (for Grow'n'Glow Yeast Plasmid Isolation kit users) on ice in an 1.5 ml Eppendorf tube.
3. Add 220 µl competent cells immediately when the cells are thawed.
4. Incubate on ice for 30 minutes.
5. Incubate at 42°C for exactly 2 minutes.
6. Place immediately on ice for 15 minutes.
7. Add 200 µl LB medium. Incubate by shaking at 37°C for 1 hour.
8. Place on ice for 5 minutes.
9. Plate the whole volume from step 7 onto a LB + 50 µg/ml Amp. plate. Incubate overnight at 37°C.

Appendix III

Order Information, Shipping & Storage

order #	description	amount
GNGK03	Grow'n'Glow One-Hybrid System:	
	pGNG2, lyophilized DNA	5 µg
	pJG4-5, lyophilized DNA	5 µg
	GNGprimer	500 pmole
	5'-PREYprimer (0.1nmole/µl)	500 pmole
	3'-PREYprimer (0.1nmole/µl)	500 pmole
	Yeast strain EGY48, glycerol stock	1 ml
	shipped on dry ice; vectors and primers store at 4°C, yeast strain store at -20°C	

Related MoBiTec Products:

order #	description	amount
GNGK01	Grow'n'Glow Two-Hybrid System "complete kit"	kit
GNGK02	Grow'n'Glow Two-Hybrid System "basic kit"	kit
ACE01	Grow'n'Glow ACE1 Two-Hybrid System "complete kit"	kit
2100-1	Grow'n'Glow Fast and Easy Yeast Transformation kit	200 transf.
2200-1	Grow'n'Glow High Efficiency Yeast Transformation kit	250 transf.
2069-1	Grow'n'Glow Yeast Plasmid Isolation kit	25 preps
2069-2	Grow'n'Glow Yeast Plasmid Isolation kit	100 preps

Grow'n'Glow Yeast and Bacterial Growth Media:

order #	description	amount
Bags for 0.5 litre medium each:		
4001-1	YPD broth bags (with 25 g for 0.5 litre medium each)	10 bags
4001-6	YPD broth bags	10 x 10 bags
order #	**description**	**amount**
4001-2	YPD agar bags (with 33.5 g for 0.5 litre medium each)	10 bags
4001-7	YPD agar bags	10 x 10 bags
4025-1	DOB* glucose bags (with 13.4 g for 0.5 litre medium each)	10 bags
4025-6	DOB glucose bags	10 x 10 bags
4025-2	DOB 2 % galactose/1 % raffinose (powder)	0.5 lb (227 g)
4025-7	DOB 2 % galactose/1 % raffinose (powder)	2.2 lb (1 kg)
4026-2	DOBA** 2 % galactose/1 % raffinose (powder)	0.5 lb (227 g)
4026-7	DOBA 2 % galactose/1 % raffinose (powder)	2.2 lb (1 kg)
4026-1	DOBA glucose bags (with 21.9 g for 0.5 litre medium each)	10 bags
4026-6	DOBA** glucose bags	10 x 10 bags
3002-1	LB medium bags (with 12.5 g for 0.5 litre medium each)	10 bags
3002-6	LB medium bags	10 x 10 bags
3002-2	LB agar bags (with 20 g for 0.5 litre medium each)	10 bags
3002-7	LB agar bags	10 x 10 bags
4510-3	CSM -HIS supplement	10 g
4511-2	CSM -URA supplement	10 g
4520-4	CSM -HIS -LEU supplement	10 g
4520-3	CSM -HIS -URA supplement	10 g
4520-5	CSM -TRP -URA supplement	10 g
4530-8	CSM -HIS -TRP -URA supplement	10 g
4540-0	CSM -HIS -LEU -TRP -URA supplement	10 g

* DOB = Drop Out Base

**DOBA = Drop Out Base with Agar